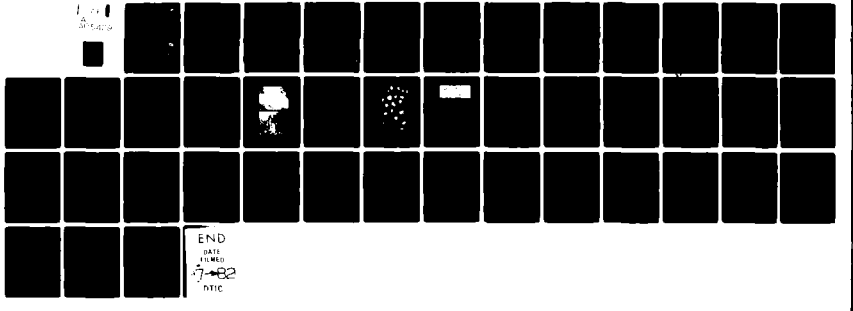
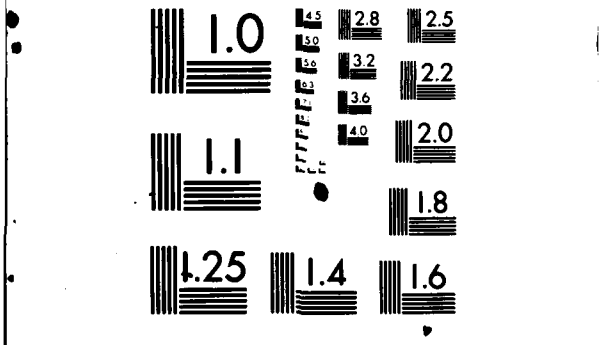
AD-A115 429 FISH AND WILDLIFE SERVICE NSTL STATION MS OFFICE OF B--ETC F/G 6/20
EFFECTS OF POLLUTANTS ON VERTEBRATE CELLS IN VITRO.(U)
1981 M W BERNS AFOSR-80-0062
UNCLASSIFIED AFOSR-TR-82-0442 NL



END
DATE FILMED
7-82
DTIC



AFOSR Grant Number 80-0062
ANNUAL SCIENTIFIC REPORT
November 1, 1980 - October 31, 1981

AFOSR-TR- 82-0442

3

AD A115429

EFFECTS OF POLLUTANTS ON VERTEBRATE CELLS IN VITRO

University of California
Irvine, CA 92717

Michael W. Berns, Ph.D.

Controlling Office: USAF Office of Scientific Research/NL
Bolling Air Force Base, DC 20332

Approved for public release;
distribution unlimited.

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFOSR-TR- 82-0442	2. GOVT ACCESSION NO. AD-A115429	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Effects of Pollutants on Vertebrate Cells in Vitro		5. TYPE OF REPORT & PERIOD COVERED Annual Report 01 Nov 1980 - 31 Oct 81 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Michael W. Berns		8. CONTRACT OR GRANT NUMBER(s) AFOSR-80-0062
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of California Department of Developmental and Cell Biology Irvine, CA 92717		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102F 2312A5
11. CONTROLLING OFFICE NAME AND ADDRESS Air Force Office of Scientific Research (NL) Bolling AFB DC 20332		12. REPORT DATE 13. NUMBER OF PAGES 6 plus attachments + D
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

laser, cell surface, mitochondria, hydrazines, calcium, cardiac, fluorescence

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Hydrazine (NH_2NH_2) and monomethyl hydrazine ($NH_2NH\,CH_3$) were examined with respect to their effects on vertebrate cell cultures. Comparative growth and morphological studies demonstrated that both compounds affect the cell surface. The technique of laser cell surface photobleaching of membrane probes is being developed as an assay of membrane fluidity changes. Considerable effort was devoted to perfecting this method by making numerous measurements on different cell types using two different probes. Cells

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

treated with Hz and MMH exhibited distinct differences from the controls with respect to the % of cells showing lateral surface mobility. Furthermore a distinct difference was observed with respect to the qualitative distribution of the surface probe in control cells versus hydrazine-treated cells. Finally, correlative electron microscopy and electrophysiology demonstrated a marked effect of MMH on cell mitochondria and CA^{++} ion flux.

B. RESEARCH OBJECTIVE

The research objectives for the period 11/1/80-10/31/81 were to continue with analysis of cells in primarily two areas: (1) studies on membrane fluidity changes, (2) studies on cytoplasmic changes. The first objective was to involve making measurements on the cell surface employing our laser fluorescence system. It was hoped that this system could be developed to assay fluidity changes of the cell membranes for different cell types when exposed to challenging agents such as hydrazines. The second objective was aimed at studying cytoplasmic target sites employing the fluorescence system as well as other methods: electrophysiology and electron microscopy. It was anticipated that this combined method attack would elucidate the basic mechanisms of hydrazine effects on cellular systems.

C. STATUS OF RESEARCH

Considerable progress was made in both of the main objective areas. In addition comparative studies between hydrazine (NH_2NH_2) and monomethyl hydrazine ($NH_2NH\ CH_3$) were performed.

With respect to the comparative studies, these studies overlapped from the previous year and the results were published (see "A" attached). These studies were designed to elucidate the cellular effects of hydrazine on four established tissue culture vertebrate cell lines (rat kangaroo kidney, Xenopus toad kidney, human diploid fibroblast, and Chinese hamster cells) and primary cultures of neonatal rat myocardial cells. Cells were exposed to hydrazine in various concentrations (0.001 to 10 mM) for varying time periods. The resulting growth and morphological data revealed a possible site of hydrazine action. In all cell lines tested, population growth was depressed by low concentrations of hydrazine (0.01 to 0.1 mM). Cell growth was initially depressed, but it eventually returned to normal log phase growth even when fresh hydrazine was added to the culture medium. At higher concentrations (0.5 to 2.0 mM), hydrazine was lethal. Most cell types first showed population growth depression at 0.01 mM hydrazine, but the lethal concentration varied with the cell type. Cultures treated with hydrazine yielded a significantly higher number of giant multinucleated cells. Autoradiography studies employing (^3H) thymidine confirmed that the large, multinucleated cells resulted from cell fusion. The increase in cell fusion in hydrazine treated cell cultures implicated the cell surface as a possible target site. Scanning electron microscopy confirmed concentration related surface differences between control and hydrazine-treated cells. Further membrane studies examining the effects of hydrazine on the contractile and intercellular spontaneous electrical activity of myocardial cells in culture indicated that hydrazine also altered these membrane-related activities in a concentration and time-dependent manner.

In the area of laser photobleaching on the cell surface, we presented prelminary results of these studies at the Ohio State Air Force Review in June of 1981 (see "B" attached). In these early studies cells were treated with a common cell surface lectin probe conconavilin A and the distribution and mobility of the fluorescent binding sites were assayed in control and hydrazine treated cells. The qualitative distribution was markedly different in Hz, MMH, and control cells. When compared to non-hydrazine treated controls, Hz treated heart cell fibroblasts showed a very marked increase in Con-A binding at the cell

peripheries, at cellular junctions, and in the ruffling edges of cell membranes. This effect was evident though greatly reduced in MMH-treated cells. It therefore appears that Hz has a greater cell surface effect, as evidenced by the distribution of Con-A binding sites.

With respect to actual mobility of the fluorescent probes, the data is summarized in Table I (next page). These data have been collected since the June Ohio meeting, and it is evident that there is an increase in receptor mobility (%M) caused by hydrazine treatment, with MMH having the greatest effect. However, it should also be noted that even though there are more sites showing mobility in Hz and MMH treated cells, the actual diffusion rates (D) do not demonstrate any consistent pattern. Because of this great variability in diffusion rates, we undertook a series of experiments in which (1) different membrane probes were used, (2) different size laser spots and bleaching times were employed. These experiments have employed the use of f- Con-A and HAF [5-(N-hexadecanoyl)] fluorescein. Since these studies are presently underway, they will be summarized in next year's annual report.

The other major area of our research was in the cytoplasm. In these studies we investigated the cytoplasmic target sites of Hz and MMH using fluorescence analysis, electron microscopy, and electrophysiology. Preliminary results presented in Ohio demonstrated that the hydrazines affected cytoplasmic mitochondria by inhibiting the fluorescent oscillating pattern of Rhodamine treated cardiac cells. These observations were further expanded, and eventually contained within a paper submitted for publication (see "C", pages 9-10 and Fig. 3). When we presented the data at the Ohio meetings, we only had a few cells in each category. As can be seen from Fig. 3 in the preprint, several hundred cells have been measured since that time. These results clearly indicate that the the hydrazines are affecting the mitochondria in addition to the cell surface.

Finally, we have performed extensive studies with electrophysiology and electron microscopy in an attempt to further elucidate the cellular effects of hydrazines. These results were also presented in Ohio (see "D" attached). However, since that time we have performed more extensive studies and are currently preparing a paper to submit to Nature. The results of these studies lead to several conclusions:

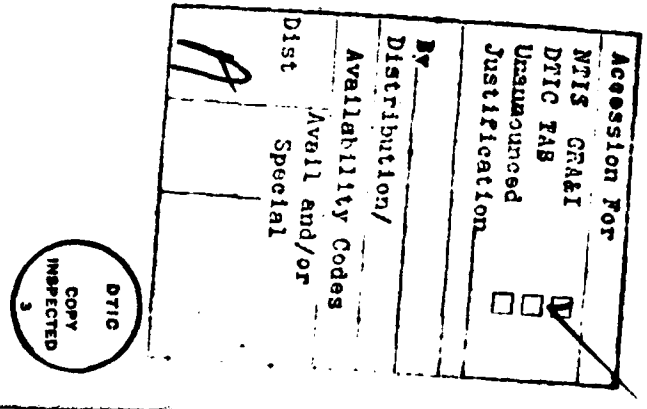
Firstly, an MMH-induced cytoplasmic $[Ca^{2+}]_i$ overload is buffered by mitochondria (presence of numerous intramitochondrial Ca^{2+} deposit granules). A residual, slightly higher than normal $[Ca^{2+}]_i$ is still presesnt in the cytoplasm as indicated by a slight electrical depolarization, increased action potential discharge frequency and contractility. This internal free Ca^{2+} concentration may not be sufficient for $G_{k(ca)}$ activation.

Secondly, prolonged mitochondrial exposure to an MMH-induced increase in cytoplasmic $[Ca^{2+}]_i$ eventually diminishes their buffering capacity due to intramitochondrial Ca^{2+} overload and impairment of their normal metabolism. Our findings show that intramitochondrial Ca^{2+} granules disappear progressively and that normal mitochondrial structure is progressively altered by MMH. Paralleling these ultrastructural changes the intracellular activity of the cells show a progressive resting membrane potential polarization (average 12mV more negative than normal). We propose that the progressive increase in K^+ conductance responsible for cell hyperpolarization is due to a gradual increase to higher

TABLE I

Evaluation of Con A Cell Surface Receptor Mobility:
Quantitation of Diffusion Constants (D) and
Percentage of Receptors Displaying Lateral Surface Mobility (%M)

Cell Type		Control	Hz Treated	MMH Treated
PTK_2	$\overline{D}$ (cm^2/sec)	3.6×10^{-4}	1.2×10^{-4}	3.5×10^{-4}
	% $\overline{M}$	22	39	49
A-6	$\overline{D}$ (cm^2/sec)	4.2×10^{-4}	4.1×10^{-4}	1.7×10^{-4}
	% $\overline{M}$	15	20	38
Heart	$\overline{D}$ (cm^2/sec)	9.9×10^{-4}	3.0×10^{-4}	6.7×10^{-4}
Fibro-blast	% $\overline{M}$	16	14	33



than physiologically normal levels of cytoplasmic [Ca^{2+}] as the mitochondria lose their buffering capacity. As demonstrated, excessive hyperpolarization in pacemaker cells may polarize them beyond pacemaker potential current activation membrane potential levels. Re-establishment of normal action potential and contraction frequency by depolarizing current injection indicates that the observed changes in electrical activity are due to an active and gradual membrane hyperpolarization induced by the proposed $[Ca^{2+}]_i$ elevation.

Our results give further evidence that: (1) mitochondria can act as Ca^{2+} buffers when the cytoplasmic concentration of Ca^{2+} increases above normal levels and (2) the level of free intracellular Ca^{2+} can affect potassium permeability in cardiac muscle. Finally, these studies directly implicate the calcium-mitochondrial system as a major target of MMH action.

D. PUBLICATIONS

Siemens, A. E., M. C. Kitzes and M. W. Berns. Hydrazine effects on vertebrate cells in vitro. Toxicol. Appl. Pharmacol. 55:378-392, 1980.

Berns, M. W., J. Aist, J. Edwards, K. Strahs, J. Girton, M. Kitzes, M. Hammer-Wilson, L.-H. Liaw, A. Siemens, M. Koonce, R. Walter, D. van dyk, J. Coulombe, T. Cahill and G. S. Berns. Laser microsurgery in cell and developmental biology. Science 213:505-513, 1981.

Siemens, A., R. J. Walter, L.-H. Liaw, and M. W. Berns. Laser stimulated fluorescence of submicron regions within single mitochondria of Rhodamine treated myocardial cells in culture. Proc. Natl. Acad. Sci., USA 79:466-470, 1982.

Siemens, A., R. Walter and M. Berns. Laser-stimulated oscillating fluorescent patterns from single mitochondria in Rhodamine 6G-treated cultured myocardial cells. ASCB Meeting, Anaheim, CA, November 9-13, 1981 (abstract).

Siemens, A. E. and M. W. Berns. Cellular effects of hydrazine and monomethyl hydrazine. (in submission).

Kitzes, M. C., L.-H. Liaw and M. W. Berns. Association of progressive myocaridial cell hyperpolarization with ultrastructural changes in mitochondria and the elevation of calcium ions. Nature (in submission).

E. PROFESSIONAL PERSONNEL

M. W. Berns, Ph.D.

M. C. Kitzes, Ph.D. (no charge consultant)

L.-H. Liaw, M.S. (no charge consultant)

A. Siemens, B.S.

Man-Hung Chow, B.S.

R. J. Walter (summer salary only - graduate student)

J. Coulombe (summer salary only - graduate student)

F. INTERACTIONS

1. Ultrastructural and electrophysiological effects of hydrazines on cells in vitro. Review of Air Force Sponsored Basic Research in Environmental Toxicology. Columbus, Ohio, June 2-3, 1981. (M. W. Berns)

2. Fluorescence studies of hydrazine treated cells in vitro. Review of Air Foirce Sponsored Basic Research in Environmental Toxicology. Columbus, Ohio, June 2-3, 1981. (A. Siemens)

3. Laser-stimulated oscillating fluorescent patterns from single mitochondria in Rhodamine 6G-treated cultured myocardial cells. American Society of Cell Biology Meeting., Anaheim, CA, November 9-13). (A. Siemens)

G. NEW DISCOVERIES, INVENTIONS OR PATENT DISCLOSURES

None.

H. ADDITIONAL INSIGHT AND INFORMATION

None.

TOXICOLOGY AND APPLIED PHARMACOLOGY 55, 378–392 (1980)

Hydrazine Effects on Vertebrate Cells *in Vitro*[1]

ANN E. SIEMENS, MARGARITA C. KITZES, AND MICHAEL W. BERNS

Developmental and Cell Biology, University of California, Irvine, Irvine, California 92717

Received February 25, 1980; accepted May 29, 1980

Hydrazine Effects on Vertebrate Cells *in Vitro*. SIEMENS, A. E. KITZES, M. C., AND BERNS, M. W. (1980). *Toxicol. Appl. Pharmacol.* 55, 378–392. This study was designed to elucidate the cellular effects of hydrazine on four established tissue culture vertebrate cell lines (rat kangaroo kidney, *Xenopus* toad kidney, human diploid fibroblast, and Chinese hamster cells) and primary cultures of neonatal rat myocardial cells. Cells were exposed to hydrazine in various concentrations (0.001 to 10 mM) for varying time periods. The resulting growth and morphological data revealed a possible site of hydrazine action. In all cell lines tested, population growth was depressed by low concentrations of hydrazine (0.01 to 0.1 mM). Cell growth was initially depressed, but it eventually returned to normal log phase growth even when fresh hydrazine was added to the culture medium. At higher concentrations (0.5 to 2.0 mM), hydrazine was lethal. Most cell types first showed population growth depression at 0.01 mM hydrazine, but the lethal concentration varied with the cell type. Cultures treated with hydrazine yielded a significantly higher number of giant, multinucleated cells. Autoradiography studies employing [^{3}H]thymidine confirmed that the large, multinucleated cells resulted from cell fusion. The increase in cell fusion in hydrazine treated cell cultures implicated the cell surface as a possible target site. Scanning electron microscopy confirmed concentration related surface differences between control and hydrazine-treated cells. Further membrane studies examining the effects of hydrazine on the contractile and intercellular spontaneous electrical activity of myocardial cells in culture indicated that hydrazine also altered these membrane-related activities in a concentration and time-dependent manner.

Hydrazine (NH_2NH_2) is a hydroscopic, highly polar reducing agent (Raphaelian, 1966). This reactive compound, described as the ammono analog of hydrogen peroxide, can be converted to a variety of widely used alkyl derivatives. Both hydrazine and its derivatives are used extensively in the production of photographic developers, agricultural chemicals, and pharmaceutical products. The use of hydrazine both as an oxygen scavenger in industry and as a major component in high-energy rocket fuel cells constitutes its major commercial uses. As a result of these applications, hydrazine and its derivatives are becoming more prevalent in the environment, and their use has been criticized as a source of biological hazard.

Previous studies have detailed the hazardous effects of hydrazine and related derivatives. Hydrazine is known to effect pyrimidine-related mutations in DNA (Brown *et al.*, 1966; Brown, 1967; Gupta and Grover, 1970; Kak and Kaul, 1975), and it is easily derivatized into a number of detrimental agents which act as both toxins and carcinogens. The most studied of these agents include the toxins, hydralazine, which interfers with smooth muscle con-

[1] The U.S. Government's right to retain a nonexclusive royalty-free license in and to the copyright covering this paper, for governmental purposes, is acknowledged.

0041-008X/80/110378-15$02.00/0

traction (McLean *et al.*, 1978), phenylhydrazine, a hemolytic agent including anemia and Heinz body formation (Jain *et al.*, 1978), and monomethyl hydrazine, a metabolic inhibitor (Dost *et al.*, 1976). A carcinogenic relative of the latter derivative, dimethylhydrazine, has produced both colon and blood vessel tumors in several laboratory animals (Toth and Wilson, 1971; Toth *et al.*, 1976; Mak and Chong, 1978; Barkla and Tutton, 1978). More specifically, some of these effects are produced in isolated cellular components only under specific conditions of treatment (Brown, 1967; Kak and Kaul, 1975). The importance of these studies may be underestimated or misinterpreted without a basic understanding of the impact such compounds have on a wide variety of cell types when tested under controlled conditions.

The present study investigates the basic cellular responses of diverse vertebrate cell types *in vitro* to the compound hydrazine. The results implicate the cell membrane as one of the major targets of hydrazine action.

METHODS

Maintenance of Cell Cultures

The PTK_2 cell line, which was derived from normal adult male kidney of *Potorous tridactylus*, was obtained from the American Type Culture Collection (CCL 56). Cells were grown in minimum essential medium (Eagle) with Earle's salts supplemented with 0.085% sodium bicarbonate, 10% heat-inactivated fetal calf serum, and 0.011% pyruvic acid. Cultures were maintained at 37°C in a 5% CO_2, 95% air atmosphere in Falcon T-75 plastic flasks. Cells were enzymatically detached from the flasks as follows: the supernatant overlying the cell monolayer was aspirated from the T-75 flask and replaced with 4 ml of an enzymatic solution (0.25% pancreatin, 0.1% EDTA in a balanced salt solution, pH 7.0). Cells detached from the substrate during incubation (6 min, 37°C) and light Pasteur pipetting. The enzymatic solution was diluted out with 5 ml of medium, and the cells were pelleted from the suspension (5 min, 200*g*). The resulting supernatant was discarded, and the cells were diluted in fresh medium for plating into either T-75 flasks (4 $\times 10^5$ cells/ml in 10 ml of medium/flask) for growth curves or into Rose multipurpose chambers (50 $\times 10^3$ cells/ml, Berns *et al.*, 1972) for microscopic evaluation.

The A6 cell line (American Type Culture Collection, CCL 102), derived from primary culture of normal male toad (*Xenopus laevis*) kidney, was maintained in Eagle's minimum essential medium (Hank's salts) fortified with 10% heat-inactivated fetal calf serum, penicillin (100,000 IU/liter) and streptomycin sulfate (0.0714 g/liter) in a 5% CO_2, 95% air atmosphere at room temperature. For weekly subculturing and experimental setups, the cells were enzymatically removed from the flasks as previously described (4 min of incubation at room temperature) and plated into T-75 flasks for growth curves (4×10^5 cells/flask in 10 ml of medium) or into Rose chambers (75×10^3 cells/ml) for microscopic evaluation.

Human diploid embryonic lung cells (W138) were purchased from the American Type Culture Collection (CCL 75). These cells were maintained in Eagle's basal medium (Earle's salts) with 10% uninactivated fetal calf serum in a 5% CO_2, 95% air atmosphere. For weekly subculturing and growth curve experiments, the cells were enzymatically detached from the flask as previously described with the following modification: the cells were incubated 5 min at 37°C in an enzymatic solution consisting of 0.25% trypsin in a balanced salt solution, pH 7.0.

Chinese hamster cells (CH), also referred to as the MC-3 line, were generously provided by Dr. Joe Gray (Lawrence Livermore Laboratory, Livermore, Calif.). They were maintained in minimum essential medium (Earle's salts) supplemented with 15% heat-inactivated fetal calf serum, 4.6% NCTC-135 (GIBCO, Grand Island, N.Y.) and 60 mg/liter gentamycin sulfate (Schering Corp., Kenilworth, N.J.) in a 10% CO_2, 90% air atmosphere at 37°C in T-25 flasks (Falcon, Pittsburg, Pa.). After enzymatic detachment from flasks (incubated 5 min, 37°C), these cells were plated in Falcon T-25 flasks at 2.5×10^4 cells/flask in 5 ml medium for growth experiments.

Hydazine Stock Solutions

Hydrazine (MW 32.05, anhydrous, 97%) was supplied through the courtesy of Dr. Ronald Shank (University of California, Irvine). Stock solutions of 100 and 10 mM hydrazine were prepared by dilution into 0.01 N HCl. The stock solutions were diluted from 50- to 1000-fold in tissue culture medium just prior to application on the cultures. Control cultures received a corresponding amount of 0.01 N HCl without hydrazine in the medium. In the quantities used, the addition of 0.01 N HCl with or without hydrazine did not significantly alter the pH of the medium.

room temperature and overnight at 4°C. The coverslips were then washed in phosphate-buffered saline and mounted on glass microscope slides. Autoradiography was performed in total darkness as follows: the slides were dipped into a 33% aqueous solution of Ilford nuclear research emulsion type L-4 (batch PL-729, Ciba-Geigy Co., Ilford, Ltd., Basidon Essex, Essex, England), dried for 1 hr at room temperature and stored in a dessicant-containing, light-tight box (4°C). One week later, the slides were developed using Kodak D-19 developer (5 min), 1% aqueous acetic acid stop (15 sec), Kodak fixer (5 min), and water rinse (20 min); all solutions were used at 18°C. The autoradiographs were stained in a filtered, saturated aqueous methylene blue solution (15 min) and destained with water. Permanent mounts were made of the slides after a series of ethanol dehydrations.

Electron Microscopy

Xenopus cells (75×10^3 cells/ml) were plated into Rose chambers in medium containing either 0.01 N HCl with hydrazine (final experimental culture hydrazine concentrations from 0.01 to 1.0 mM) or corresponding amounts of 0.01 N HCl alone (control cultures). Fresh medium containing hydrazine was added at 48 hr. After 3 days, the cells were fixed in medium with 2% glutaraldehyde (30 min at room temperature, and overnight at 4°C), and the adherent cells on the glass coverslips were processed for SEM according to the procedures described by Cohen *et al.* (1968). The coverslips were rinsed 5 min in phosphate-buffered saline and run through a series of 5-min dehydrations in aqueous ethanol (50–100%). This was followed by a series of 10-min ethanol–freon dehydrations (30–100% Freon 113), critical point drying (Omar SPE-900EX) utilizing Freon 113 and gold evaporation (Technis Hummer II, 3 min at 10 V). Coverslip specimens were mounted with silver paint on aluminum studs and analyzed on a Hitachi HS500 scanning electron microscope (15–20 kV, tilt angle of 30–55°). Random samples of cells on the coverslips were examined with regard to the quantity of cell surface projections (light, moderate, or heavy surface detail) that each cell displayed. Tabulations of the number of cells displaying each type of surface detail were prepared from each specimen by an investigator who was unaware of which specimen corresponded to each hydrazine concentration. Evaluation of the observed data was verified with Student's *t* test (confidence level at least 95%).

Heart Culture/Electrophysiology

Neonatal rat (1–2 days old) ventricular cells were cultured in Rose chambers according to the methods previously described (Kitzes and Berns, 1979). Glass micropipet microelectrodes filled with 2.7 M K-citrate were utilized for intracellular recording. Electrodes were selected with resistances between 20 and 50 megohms. Individual contracting myocardial cells were selected for impalement by observation through a Nikon inverted-phase microscope. The microelectrode was carefully lowered into the selected cell using a de Fonbrune pneumatic micromanipulator, and the electrical properties of the cell were recorded and analyzed according to the procedures described earlier (Kitzes and Berns, 1979). Recordings were made both before exposure to hydrazine and during the indicated times after the medium bathing the cells was replaced by medium supplemented with 0.01, 0.1, or 1.0 mM hydrazine.

RESULTS

Figure 1 demonstrates the basic dose-related growth response of four cell types to hydrazine concentrations of 0.01 to 10 mM. Although the different cell types expressed varying degrees of sensitivity toward hydrazine, all cell types showed several common responses. Hydrazine was cytotoxic to all populations tested at dosages ranging from 0.5 to 4 mM, depending on the cell type. At lower concentrations, hydrazine produced a dose-dependent suppression (but not complete inhibition) of population growth with 0.01 to 1.0 mM being the threshold range of response for most cell types tested. For each cell type, there appeared an optimum dose (OD) of hydrazine which initially suppressed population growth from control levels and yet allowed the treated population to recover to log phase growth. This dosage appeared to be 1.0 mM for PTK_2, 1.0 mM for A6, 0.1 mM for WI38, and 0.05 mM for CH cells. The return of OD-treated populations to log phase growth implies that either the action of hydrazine on a cell may be short-lived or that nonhydrazine sensitive cells in the population are being selected for.

In addition to being dose dependent (Fig. 1), the growth suppression seen in both A6 and PTK_2 cell types appeared to be related to the length of time of hydrazine exposure.

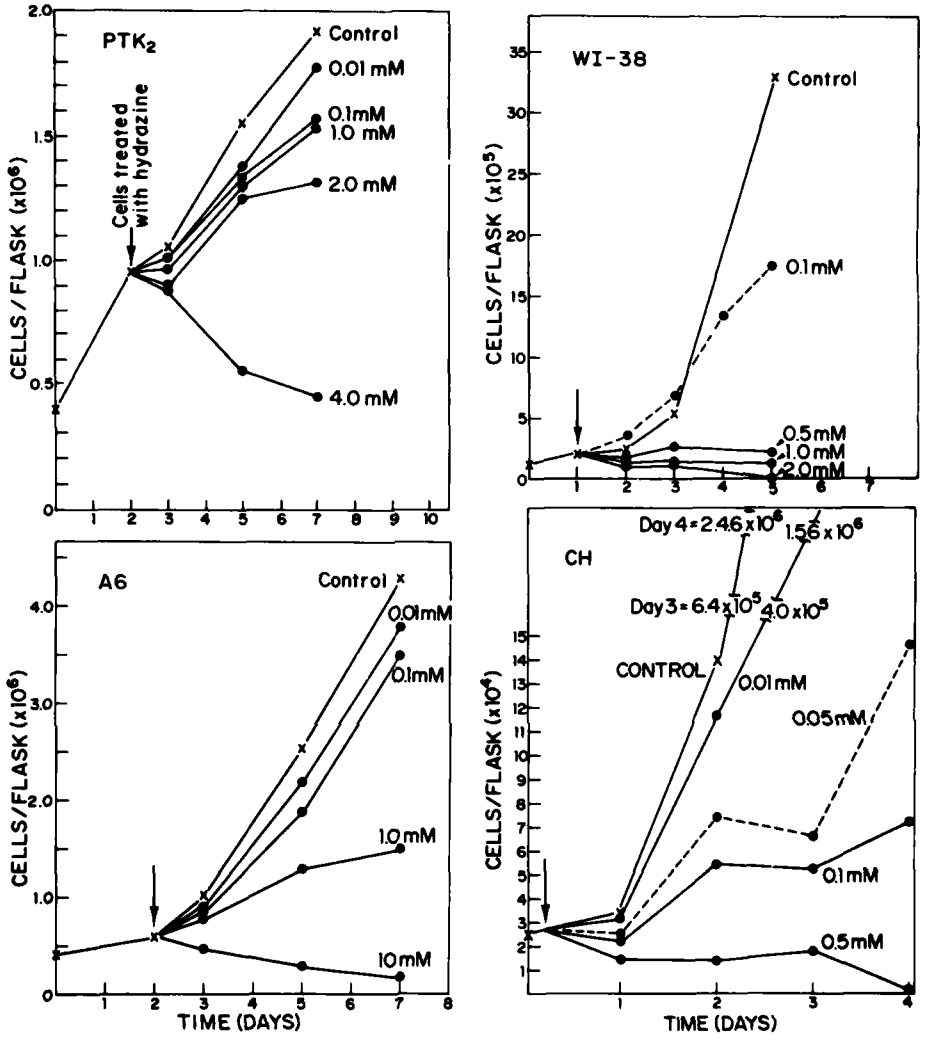


FIG. 1. Growth response curves of four cell lines (PTK_2, WI-38, A6, and CH) to solutions containing the indicated hydrazine concentrations. Cells were exposed to fresh hydrazine at every point along the graph throughout the duration of the experiment. Arrows indicate the point of initial treatment.

The effects caused by various exposure lengths of 1.0 mM hydrazine on PTK_2 and A6 cells are illustrated in Fig. 2. These cultures were replenished with medium containing fresh hydrazine at the initial exposure point (arrow) and at every subsequent data point up to 24 or 96 hr. The cultures designated "continuous" received fresh hydrazine-containing medium at each data point throughout the experiment. Beyond the designated time of treatment, the medium was replaced at each data point with fresh medium containing no hydrazine. The net population increase observed in cultures receiving fresh hydrazine containing medium at every point beyond induction is noteworthy. It suggests either a selection of genetically resistant cells or a physio-

logical adaptation of cells to hydrazine. It is not likely that the observed effect is due to hydrazine breakdown in the medium; if this were occurring, at least an initial depression in growth rate should be observed after exposure to each fresh hydrazine application.

To test for the selection of hydrazine resistant cells, OD-treated A6 cells which achieved log phase growth (Day 10) were replated and treated anew with fresh hydrazine (Fig. 3). The "selected" cells responded to hydrazine treatment with severe population growth suppression similar to the untreated "naive" cells. The difference between the growth rates of "selected" and "naive" cells was insignificant when compared to the control growth rate. There was no appreciable difference between growth rates of untreated "naive" and untreated "selected" controls (data not shown).

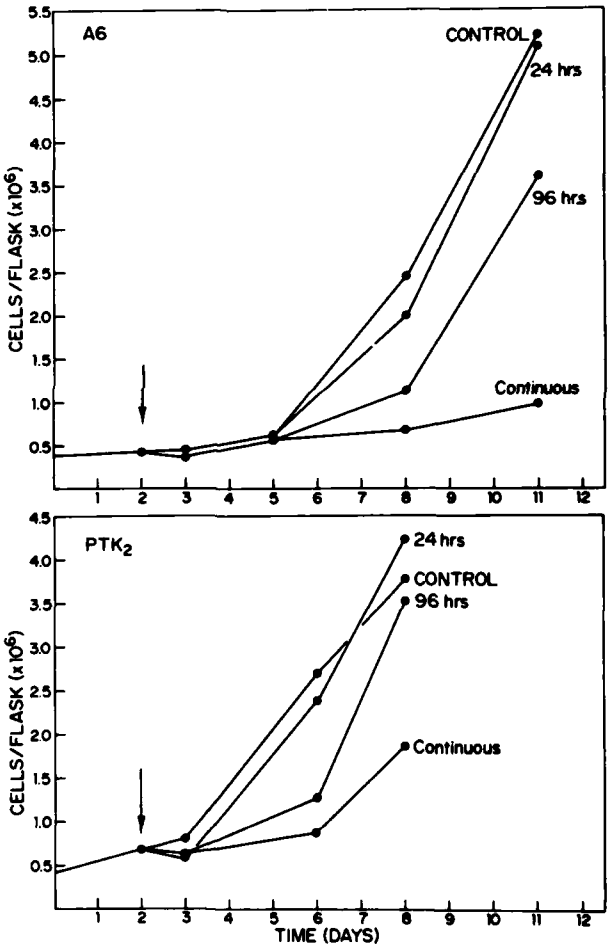


FIG. 2. Growth curves of A6 cells and PTK_2 cells treated with 1.0 mM hydrazine for varying exposure periods. Established cultures received medium containing fresh hydrazine at each point from the initial point of treatment (indicated by arrow) up to the designated time—24 or 96 hours, or throughout the duration of the experiment (continuous). Beyond the indicated treatment, the cultures received fresh medium without hydrazine at each subsequent point.

These results indicate that no genetic selection was operating in the observed recovery from hydrazine-induced growth suppression. Further attempts to detect hydrazine-induced genetic mutation in several cell lines via ouabain resistance and growth in sloppy agar were completely unsuccessful.

Besides unsuccessful mutation assays, several other experiments were performed to detect hydrazine-induced cellular anomalies which would account for the behavior of the experimental populations. Examination of earlier Coulter counter data of control and hydrazine treated populations revealed an apparent cell size increase in the experimental cultures (Fig. 4). In a typical dose response growth curve of 72-hr-treated populations, the experimental cultures had a measurably larger mean cell volume than the control cultures (plots of cell populations are taken from Day 5 samples of control and 1.0 mM-treated A6 and PTK_2 cells shown in Fig. 1). Further

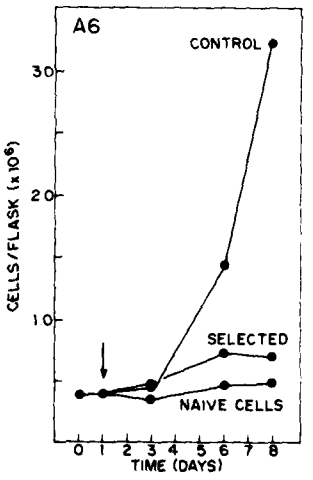


FIG. 3. Effects of 1.0 mM hydrazine on the growth rates of two subpopulations of A6 cells: "selected" cells, which had been grown to confluency in the presence of 1.0 mM hydrazine, and "naive" or unselected A6 cells. Both populations were treated with hydrazine throughout the experiment from initial time of exposure (indicated by arrow).

TABLE 1

INCIDENCE OF MULTINUCLEATION OCCURRING IN BOTH CONTROL PTK_2 CULTURES AND EXPERIMENTAL CULTURES TREATED FOR 2 DAYS WITH 1.0 mM HYDRAZINE

	Uni-nucleated cells (%)	Multi-nucleated cells (%)	Total No. cells
Control cultures	92	8	170
Experimental cultures (hydrazine treated)	78	22[a]	159

[a] Hydrazine-treated cultures had significantly more multinucleate cells than did controls (at least 95% confidence).

microscopic analysis of PTK_2 cells revealed significantly more multinucleate cells in 1.0 mM hydrazine treated populations than in control populations (Table 1, Fig. 5). This threefold increase in multinucleation resulted in each of triplicate experiments performed.

The increase in cellular multinucleation suggests that hydrazine may act to produce either abnormal mitosis resulting in multiple nuclei or cell surface alterations promoting cell fusion. Experiments were undertaken to test for both possibilities in 1.0 mM-treated cell populations. Because PTK_2 cells remain perfectly flat throughout mitosis (Rattner and Berns, 1974), it was possible to carefully observe mitosis by light microscopy. Studies employing still and time lapse photography revealed no difference in mitotic abnormalities between control and treated populations. However, evidence for increased cell fusion in hydrazine-treated populations implicated the cell surface as a possible target site for hydrazine action (Table 2). In a mixed population of PTK_2 cells having either regular or ^3H-tagged nuclei, the presence of multinucleated cells containing both types of nuclei (tagged and untagged) suggested that cell fusion was occurring. There was a fivefold increase of these types of cells in the hydrazine-treated populations as compared to the con-

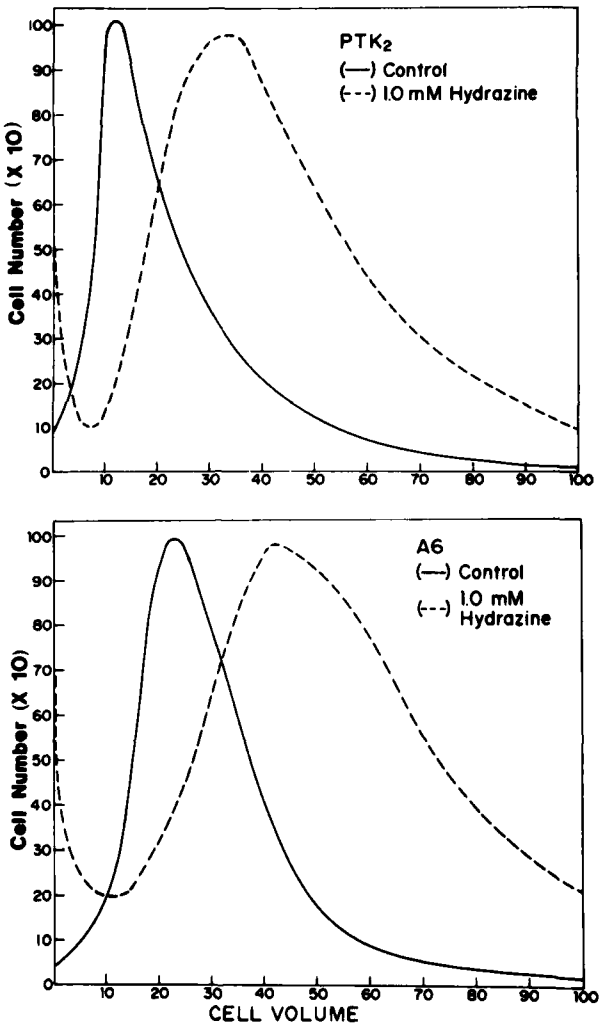


FIG. 4. Effect of 1.0 mM hydrazine on the relative distribution of individual cell volumes from PTK_2 and A6 suspended cell cultures (from Fig. 1, Day 5) as determined by Coulter counter–channelyzer plots. Abscissa represents 100 graded relative cell volume channels. Ordinate illustrates the number of cells in a population fitting a certain channel size; maximum cell number per channel = 1000.

trol populations (Fig. 6 and Table 2). Furthermore, the quantity of these cells comprising the entire multinucleate population of a culture was significantly higher (1.5-fold) in the hydrazine-exposed cultures than in control cultures.

To further investigate membrane effects, cell surface morphologies of hydrazine-treated and control A6 cells were studied (Fig. 7). Table 3 presents data showing the effect of hydrazine on the cell surface. It is clearly evident that there is a significant, inverse relationship between the hydrazine concentration a cell received and the amount of SEM detectable cell surface projections it displayed. Furthermore,

FIG. 5. Phase contrast micrographs of PTK_2 control cultures (A) and 72 hr, 1.0 mM hydrazine-treated cultures (B). Magnification, 2200×.

TABLE 2

INCIDENCE OF CELL FUSION OCCURRING WITHIN THE MULTINUCLEATED POPULATIONS IN BOTH CONTROL PTK_2 CULTURES AND EXPERIMENTAL CULTURES TREATED FOR 3 DAYS WITH 1.0 mM HYDRAZINE

	Uni-nucleated cells (%)	Multi-nucleated cells (%)	Similarly labeled nuclei[a] (%)	Dis-similarly labeled nuclei[b] (%)
Control cultures	95	5	4	1
Experimental cultures	85	15	10	5[c]

[a] Refers to multinucleated cells containing either all normal or all [^{3}H]thymidine-tagged nuclei.

[b] Refers to multinucleated cells containing both normal and [^{3}H]thymidine-tagged nuclei.

[c] Hydrazine-treated cultures had significantly more dissimilarly labeled multinucleated cells than controls did (confidence level at least 95%).

this inverse relationship is concentration dependent, paralleling the results of the growth response curves.

Since the cell fusion data and the SEM data implicated the cell membrane as a primary site of hydrazine action, additional studies were undertaken to examine the effect of hydrazine (0.01 to 1.0 mM) upon membrane-associated electrical and contractile activities of neonatal rat myocardial ventricular cells in culture.

A typical intracellular recording of spontaneous action potentials recorded in a rhythmically contracting heart cell is shown in Fig. 8a. The resting membrane potential is approximately −60 mV, and the action potentials occur at a rate of approximately 1 per second. In Figs. 8b and c are shown typical recordings 10 and 20 min following exposure of the culture to 0.01 mM hydrazine. Three effects are apparent: (1) a depolarization of the resting membrane potential, (2) a reduction of action potential amplitude, and (3) a disruption of discharge rhythmicity. These electrical changes were accompanied by a disruption of the rhythmic contractile behavior of the cell. However, electrical activity returned to almost normal status 30 min after cells initially received medium containing 0.01 mM hydrazine (Fig. 8d).

Exposure to 0.1 mM hydrazine (Fig. 9b) resulted in (1) a more severe reduction in membrane potential that is still evident 15 min after exposure, (2) a complete absence of action potential discharge, and (3) arrhythmic baseline activity. During this time, the cell was not contracting. At this hydrazine concentration, the cells resumed normal electrical and contractile activity after 45 min in the hydrazine-supplemented medium (Fig. 9c). Cells exposed to 1.0 mM hydrazine (Fig. 10) did not recover after 45 min to 1 hr in the experimental medium. At this time (Fig. 10), only very aberrant electrical activity accompanied by occasional small and atypical contractile activity was observed. However, these cells resumed normal electrical and contractile activity 15 min after the hydrazine-containing medium was replaced with normal medium (data not shown).

DISCUSSION

The purpose of this study was to examine the basic cellular effects of hydrazine. Previous studies have linked hydrazine and its derivatives with carcinogenic and mutagenic effects. Carcinogenic effects of the hydrazine analog 1,2-dimethylhydrazine have been reported *in vivo* in studies of rodent intestines (Barlka and Tutton, 1977, 1978; Jacobs, 1977; Mak and Chong, 1978; Richards, 1977; Sunter *et al.* 1978; Toth *et al.* 1976) and blood vessels (Toth and Wilson, 1971). Hydrazine has been shown to mutate DNA from a variety of sources (Raphalian, 1966; Dave, 1977; Brown, 1967; Brown *et al.*, 1966; Gupta and Grover, 1970; Kak and Kaul, 1975; Kimball, 1977; Kimball and Hirsch, 1975, 1976; Lemontt, 1977). These effects appear as the result of the chemical treatment of selected target tissues under specific conditions. In this study, however, a diverse variety of tissue culture cell types were employed to ascertain the basic cellular effects of hydrazine.

FIG. 6. PTK$_2$ cell cultures, stained after autoradiography with methylene blue, from control (A) and 32 hr, 1.0 mM hydrazine-treated (B) cultures. Cells containing both labeled and unlabeled nuclei were formed by cell fusion. Magnification, 1900×.

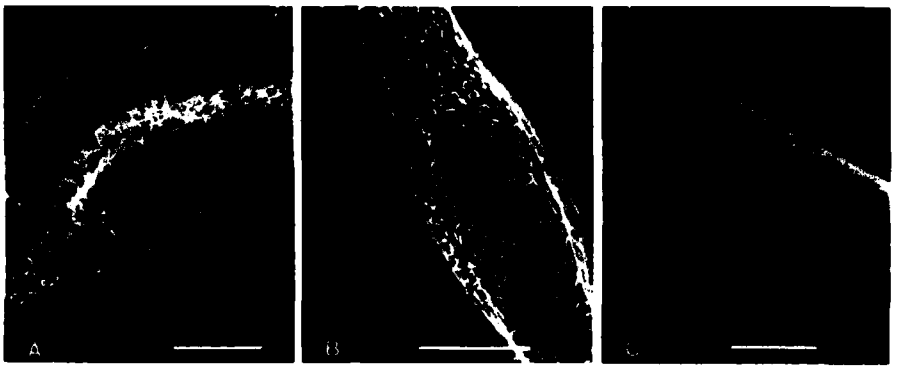


FIG. 7. Scanning electron micrographs of A6 cells which typify (A) heavy, (B) moderate, and (C) light cell surface projections. Bar, 5 μm.

The noted hydrazine-induced growth effects shown in this study could be classified into two basic types of responses. First of all, the cells demonstrated a concentration-dependent response to hydrazine treatment (Figs. 1, 8, 9, 10 and Table 3). In these studies, different hydrazine concentrations, usually spanning three or four orders of magnitude, caused effects ranging from imperceptible cellular changes to lethality. Within this concentration range, the cells could survive, proliferate, and function. Second, in the growth response curves, the growth rate remained depressed while cells received intermittent rechallenges to the higher hydrazine concentrations; however, the growth rates resumed normal log phase levels when hydrazine-containing medium bathing the cells was replaced with normal medium (Figs. 2, 3, Control—see Methods). At the lower concentrations of hydrazine treatment, the cells exhibited an

TABLE 3

EFFECTS OF VARIOUS HYDRAZINE DOSAGES ON SEM DETECTABLE XENOPUS CELL SURFACE PROJECTION

Hydrazine concentration (mM)	Cells with light surface projection (%)	Cells with moderate surface projection (%)	Cells with heavy surface projection (%)	Total No. cells sampled
0	24	37	39	196
0.01	35.5	31.5	33	186
0.1	60	31.5	8.5	188
1.0	79	17	4	203

Note. Statistical analysis verified a significant difference in the quantity of light or heavy cell surface projection present between populations of control cultures and populations receiving either 0.1 or 1.0 mM hydrazine (confidence level at least 95%).

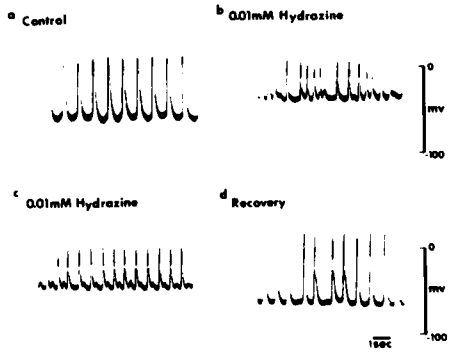


FIG. 8. Effects of exposure to 0.01 mM hydrazine on the spontaneous intracellular electrical activity of rhythmically contracting myocardial cells in culture: (a) Control activity (no exposure to hydrazine); electrical activity after exposure to 0.01 mM hydrazine for (b) 10, (c) 20, and (d) 30 min.

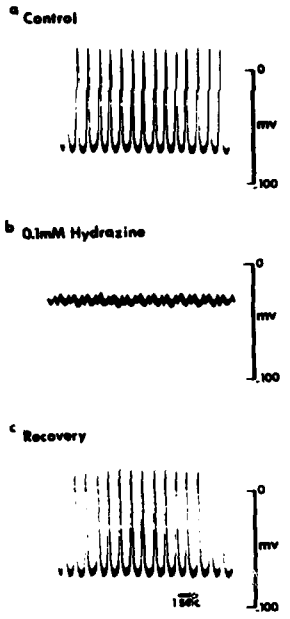


FIG. 9. Effects of exposure to 0.1 mM hydrazine (a) control; (b) 15 min after culture received 0.1 mM hydrazine solution; (c) 45 min after culture received 0.1 mM solution.

initial depression in growth, but, upon re-exposure to fresh hydrazine, they appeared to attain a normal growth rate. Likewise in heart cell cultures, the aberrant electrical and contractile effects produced by the presence of 1.0 mM hydrazine abated when these cells were washed and bathed in normal medium. In the presence of low hydrazine concentrations, treated heart cells appeared to show some recovery from the observed effects (Figs. 8, 9).

The mechanism whereby treated cells rebound in the presence of fresh hydrazine is unclear. It seems possible that this "tolerance" or "recovery" may be due to an increased capacity of exposed cells to inactivate hydrazine or its effectual metabolite. The production of "tolerant" cells in the presence of hydrazine could also suggest the selection of genetically resistant cells or the alteration of an affected organelle to a hydrazine-refractory state. Regardless of the mechanism, it appears that hydrazine evokes some form of selection or tolerance. This observation is supported by data showing that entire populations, rather than a few selected cells, seem altered by hydrazine treatment (Figs. 3, 4). Furthermore, Fig. 3 indicates that, in the tissues studied, hydrazine acted in a disruptive but nonmutational manner. It therefore seems logical that hydrazine may elicit these concentration dependent and reversible responses by actively and nonmutagenically interfering with a common cellular site in a wide spectrum of cell types. The SEM and electrophysiological studies, which were done on very different cell types, suggest that the cell surface may be a common target site of hydrazine action. Furthermore, the increase of multinucleation by cell fusion seen in hydrazine-treated cultures also indicates that the cell surface is a major site of this compound's action.

The above observation is further supported by the literature in which hydrazine derivatives have been shown to elicit a wide variety of effects in studies performed on biological membranes (Balduini *et al.*, 1977; Barkla and Tutton, 1977; Braun and Wolfe,

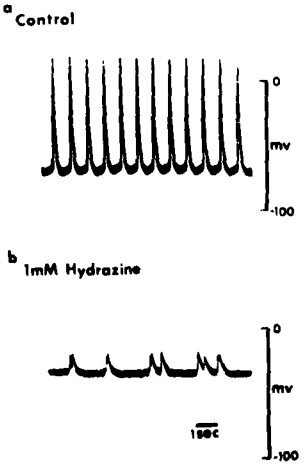


FIG. 10. Effects of exposure to 1.0 mM hydrazine (a) control activity, (b) aberrant electrical activity after 1 hr in 1.0 mM hydrazine solution.

1977; Caroni, 1977; Jain and Subrahmanyam, 1978; Jain *et al.*, 1977, 1978; Katsumata *et al.*, 1977; McLean *et al.*, 1978; Tsau *ete al.*, 1977; Walter *et al.*, 1978; Zimmer, 1977). The observations of these numerous studies support the view that the hydrazine-induced cellular effects seen in our data stem from membrane interactions with this compound. Furthermore, it seems logical that such a strong reducing agent as hydrazine would directly affect the cell structure it first contacts.

ACKNOWLEDGMENT

This research was supported by U.S. Air Force Grant AFOSR-77-3136.

REFERENCES

BALDUINI, C. L., RICEVUTTI, G., SOSSO, M., ASCARI, E., AND BALDUINI, C. (1977). Membrane sialic acid and behavior in vivo of rabbit "stress" macroreticulocytes. *Hoppe Seyler's Z. Physiol. Chem.* **358**, 1143–1148.

BARKLA, D. H., AND TUTTON, P. J. M. (1977). Surface changes in the descending colon or rats treated with dimethylhydrazine (DMH). *Cancer Res.* **37**, 262–271.

BARKLA, D. H., AND TUTTON, P. J. (1980). Ultrastructure of 1,2,dimethylhydrazine-induced adenocarcinomas in rat colon. *J. Nat. Cancer Inst.*, 1291–1299.

BERNS, M. W., FLOYD, A. D., ADKISSON, K., CHENG, W. K., MOORE, L., HOOVER, G., USTICK, K., BURGOTT, S., AND OSIAL, T. (1971). Laser microirradiation of the nucleolar organizer in cells of the rat kangaroo (*Potorous tridactylis*). Reduction of nucleolar number and production micronucleoli. *Exp. Cell Res.* **75**, 424–432.

BRAUN, R., AND WOLFE, G. (1977). The influence of N-methyl-N-β-chloroethyl hydrazines on the mitotic index of Ehrlich ascites tumor cells and the leukkopoiesis of the mouse. *Experientia* **33**, 66–67.

BROWN, D. M. (1967). The reaction of hydrazine with pyrimidine bases. In *Methods in Enzymology* (L. Grossman and K. Moldave, eds.), Vol. 12, pp. 31–34. Academic Press, New York.

BROWN, D. M., MCNAUGHT, A. D., AND SCHELL, P. (1966). The chemical basis of hydrazine mutagenesis. *Biochem. Biophys. Res. Commun.* **24**, 967–971.

CARONI, P., GAZZOTTI, P., VUILLEUMIER, P., SIMON, W., AND CARAFOLI, E. (1970). Ca^{2+} transport mediated by a synthetic neutral Ca^{2+} ionophore in biological membranes. *Biochem. Biophys. Acta* **470**, 437–445.

COHEN, A. L., MARLOW, D. P., AND GARNER, G. E. (1968). A rapid critical point method using fluorocarbons (freons) as intermediate and transitional fluids. *J. Microsc.* **7**, 331–342.

DAVE, C., EHRKE, M. J., AND MIHICH, E. (1977). Studies on the structure-activity relationship among aliphatic and aromatic bisguanylhydrazones and some related compounds. *Chem.-Biol. Interact.* **16**, 57–68.

DOST, G. N., JOHNSON, D. E., AND WANG, C. H. (1976). Metabolic toxicity of simple hydrazines: monomethyl hydrazine (MMH). *Toxicol. Appl. Pharmacol.* **49**, 225–236.

GUPTA, A. K., AND GROVER, N. S. (1970). Hydrazine induced breaks in chromosomes of *Vicia faba*. *Mutat. Res.* **10**, 519–520.

JACOBS, M. M. (1977). Inhibitory effects of selonium on 1,2-dimethylhydrazine and methylazoxymethanol colon carcinogenesis. *Cancer* **40**, 2557–2564.

JAIN, S. K., CHAUHAN, U. P. S., MAZUMDAR, S., AND SUBRAHMANYAM, D. (1978). Effect of hydrocortisone on phenylhydrazine-induced Heinz-body anemia. *Canad. J. Physiol. Pharmacol.* **56**, 677–679.

JAIN, S. K., MAJUMDAR, S., AND SUBRAHMANYAM, D. (1977). Effects of phenylhydrazine on the plasma lipids of albino rat. *Naunyn-Schneideberg's Pharmacol.* **298**, 75–77.

JAIN, S. K., AND SUBRAHMANYAM, D. (1978). On the mechanism of phenylhydrazine-induced hemolytic anemia. *Biochem. Biophys. Res. Commun.* **82**, 1320–1324.

KAK, S. N., AND KAUL, B. L. (1975). Mutagenic activity of hydrazine and its combinations with maleic hyarazide and x-rays in barley. *Cytobios* **12**, 123–128.

KATSUMATA, Y., TANAKA, F., HAGIHARA, M., AND YAGI, K. (1977). Changes in membrane fluidity of erythrocytes during cell maturation. *Biochem. Biophys. Res. Commun.* **78**, 609–614.

KIMBALL, R. F. (1977). The mutagenicity of hydrazine and some of its derivatives. *Mutat. Res.* **39**, 111–126.

KIMBALL, R. F., AND HIRSCH, B. F. (1975). Tests for the mutagenic action of a number of chemicals on *Haemophilus influenza* with special emphasis on hydrazine. *Mutat. Res.* **30**, 9–20.

KIMBALL, R. F., AND HIRSCH, B. F. (1976). Fixation loss of hydrazine-induced premutational damage in *Haemophilus influenza*. *Mutat. Res.* **36**, 39–48.

KITZES, M. C., AND BERNS, M. W. (1979). Electrical activity of rat myocardial cells in culture: La^{3+}-induced alterations. *Amer. J. Physiol. Cell Physiol.* **6**, C87–C95.

LEMONTT, J. F. (1977). Mutagenesis of yeast by

hydrazine: Dependence upon post-treatment cell division. *Mutat. Res.* **43**, 165–178.

LYNCH, G. P., SMITH, D. F., COPE, R. C., AND FISHER, M. (1978). Heinz body formation in calf erythrocytes. *Dairy Sci.* **61**, 1161–1166.

MAK, K. M., AND CHONG, W. W. (1978). Inhibition of DNA synthesis by 1,2-dimethylhydrazine and methylazoxymethanol acetate in rabbit colon muscosa in organ culture. *J. Nat. Cancer Inst.* **61**, 799–804.

MCLEAN, A. J., BARRON, K., DU SOUICH, P., HAEGELE, K. D., MCNAY, J. L., CARRIER, O., AND BRIGGS, A. (1978). Interaction of hydrazine and hydrazone derivatives with contractile mechanisms in rabbit aortic smooth muscle. *J. Pharmacol. Exp. Therap.* **205**, 418–425.

RAPHAELIAN, L. A. (1966). Hydrazine and its derivatives. In *Encyclopedia of Chemical Technology* (H. F. Mark, J. J. McKetta, Jr., D. F. Othmer, and A. Standen, eds.), Vol. 11, pp. 164–196. Interscience, New York.

RATTNER, J. B., AND BERNS, M. W. (1974). Light and electron microscopy of laser microirradiated chromosomes. *J. Cell Biol.* **62**, 526–533.

RICHARDS, T. C. (1977). Early changes in the dynamics of crypt cell populations in mouse colon following administration of 1,2-dimethylhydrazine. *Cancer Res.* **37**, 1680–1685.

SUNTER, J. P., APPLETON, D. R. WRIGHT, N. A., AND WATSON, A. J. (1978). Pathological features of the colonic tumors induced in rats by the administration of 1,2-dimethylhydrazine. *Virchows' Arch. B* **29**, 211–223.

TOTH, B., MALICK, L., AND SHIMIZU, H. (1976). Production of intestinal and other tumors by 1,2-dimethylhydrazine dihydrochloride in mice. *Amer. J. Pathol.* **84**, 69–80.

TOTH, B., AND WILSON, B. (1971). Blood vessel tumorigenesis of 1,2-dimethylhydrazine dihydrochloride (symmetrical). I. Gross, light, and electron microscopic descriptions. *Amer. J. Pathol.* **64**, 585–600.

TSAU, D., COLTON, D. C., CHANGE, J. S., BUCK, R. L., HUDSON, B. G., AND CARRAWAY, K. L. (1977). Alterations of RBC membranes from phenylhydrazine-treated rabbits. *Biochim. Biophy. Acta* **469**, 61–73.

WALTER, H., KROB, E., AND ASCHER, G. S. (1978). Abnormal membrane lipid-related properties during maturation of reticulocytes from severely anemic rats as measured by partition in two-polymer aqueous phases. *Brit. J. Haematol.* **39**, 391–397.

ZIMMER, G. (1977). Carbonylcyanide p-trifloromethoxyphenylhydrasone-induced change of mitochondrial membrane structure revealed by lipid and protein spin labeling. *Arch. Biochem. Biophys.* **181**, 26–32.

"B"

Fluorescence Studies of Hydrazine Treated Cells In Vitro

Ann Elizabeth Siemens, Robert J. Walter, and Michael W. Berns
University of California, Irvine, CA 92717

In order to elucidate the effects of hydrazine and monomethyl hydrazine on vertebrate cells in vitro further, fluorescent studies were undertaken on the two cell components shown to be targets of the hydrazines: the cell membrane and the mitochondria.

Hydrazine treated cells were examined using the common cell surface receptor probe, the fluoresceinated lectin, concanavilin A. Agents that effect the cell surface often affect the distribution of Con-A binding sites. When compared to non-hydrazine treated controls, Hz treated heart cell fibroblasts showed a very marked increase in Con-A binding at the cell peripheries, at cellular junctions, and in the ruffling edges of cell membranes. This effect was evident though greatly reduced in MMH-treated cells. It therefore appears that Hz has a greater cell surface effect, as evidenced by the distribution of Con-A binding sites; internally, however, the greater mitochondrial effects are produced by MMH, as evidenced by the TEM studies in the previous paper.

In order to characterize the difference between the two hydrazines further and to elucidate the mitochondrial effect, fluorescent measurements were made on pre-selected individual mitochondria using a focused laser beam. Cardiac cells were first treated with the mitochondrial fluorochrome Rhodamine 6G. The cells were next exposed to appropriate dosages of Hz and MMH. A blue 442 nm helium cadmium laser was then focused to a 1μm spot inside a pre-selected single mitochondrion, and the fluorescence emission was recorded over time using a photon sensitive photomultipler attached to a multichannel analyzer. Control (non-hydrazine treated) cells exhibited a cyclical fluorescent pattern that consisted of bursts of intense fluorescence followed by a major decrease in fluorescence. It was normal to observe alternating periods of intense fluorescence followed by weak fluorescence over a 5-10 minute period. Cells treated with the parental hydrazine compound exhibited an initial intense fluorescent emission followed by a sudden decrease in fluorescence, as did the controls. However, there was no cyclical pattern of recovery and decrease. Instead, after the initial burst of fluorescence there was only erratic, weak, small fluorescent peaks. Treatment with MMH completely eliminated any significant change in fluorescent intensity following the initial fluorescent peak.

In conclusion, both the cell surface fluorescent patterns and the mitochondrial oscillating fluorescent decays demonstrate a distinct effect of the hydrazines on the cells. In addition, the data in both assay systems indicate a difference in effect of the two hydrazines studied.

"C"

CELLULAR EFFECTS OF HYDRAZINE AND MONOMETHYL HYDRAZINE

Anne E. Siemens and Michael W. Berns

Developmental and Cell Biology
University of California
Irvine, California 92717

The present study extended earlier work to elucidate the cellular mode of action of the hydrazines. In particular the compounds hydrazine (Hz) and monomethyl hydrazine (MMH) were compared with respect to effects on (1) cell growth, (2) cell surface microvilli, and (3) fluorescence intensity of single mitochondria stained with Rhodamine 6G. Both compounds exhibited a dose dependent depression on the growth of Xenopus (A6) frog kidney cells, though MMH was more cytotoxic (2 mM) than was Hz (10 mM). Both compounds induced a reduction in cell surface microvilli; however, Hz was more effective than MMH. With respect to alteration in mitochondrial fluorescence of rat myocardial cells, both compounds affected the pattern when compared to controls, though the effects of Hz and MMH do appear to differ. The results reported here confirm that both Hz and MMH affect (1) cell growth, (2) the cell surface, and (3) cellular mitochondria. The degree to which these compounds affect the above, is different.

Cellular Effects of Hydrazine and Monomethyl Hydrazine, SIEMENS, A. E., AND BERNS, M. W. (1982).

Because of the widespread use of hydrazines as a major fuel propellant and as an oxygen scavenger in industry, the biological effects of these compounds is of significant concern. Studies have dealt with the mutagenecity of the hydrazines (Kimball, 1977, Rogers and Back, 1981), their carcinogenetic potenteial (Barkla and Tutton, 1978)), and their metabolic toxicity (Dost et al., 1979). In addition, hydrazines have been shown to have teratogenic effects on mammalian (Schiller et al., 1979) and amphibian embryonic development (Greenhouse, 1976).

Since most of the earlier studies focused on the genetic and biochemical effects of hydrazines, the present studies were undertaken to elucidate the cellular and subcellular sites of hydrazine action. The parental hydrazine compound (NH_2NH_2) was shown to have a dose dependent growth inhibitory effect on a wide variety of vertebrate cell cultures. In addition, it was demonstrated using electrophysiology, scanning electron microscopy, and thymidine autoradiography, that a primary cellular site of hydrazine action was the cell membrane (Siemens, et al., 1980).

Because studies by other investigators (Dost et al., 1979, Rogers and Back, 1981, Shiller et al., 1979) have demonstrated that the effects of hydrazine and its derivatives vary from compound to compound, we have compared the biological effects of the parental hydrazine (Hz) and one of its most commonly used methyl derivatives, monomethyl hydrazine (MMH). In addition to looking at cell growth and cell surface membrane effects, studies have been expanded to examine the effects of hydrazines on internal membranous organelles such as mitochondria.

MATERIALS AND METHODS

Cell Culture

Two cell culture systems were used in the present study; an established kidney cell line A6 from the South African clawed toad Xenopus laevis, and primary cultures of newborn rat heart myocardial cells. The A6 cell line (American Type Culture Collection, CCL 102), derived from primary culture of normal male toad (Xenopus laevis) kidney, was maintained in Eagle's minimum esesential medium (Hank's salts) fortified with 10% heat-inactivated fetal calf serum, penicillin (100,000 IU/liter) and streptomycin sulfate (0.0714 g/liter) in a 5% CO_2, 95% air atmosphere at room temperature. For weekly subculturing and experimental setups, the cells were enzymatically removed from the flasks (4 min. of incubation at room temperature) and plated into T-75 flasks for growth curves (4×10^5 cells/flask in 10 ml of medium) or into Rose chambers (75×10^3 cells/ml) for microscopic evaluation.

Neonatal rat myocardial cells were established from 2-4 day old rats according to methods described elsewhere (Kitzes and Berns, 1979). Ventricles were minced into 1-2 mm^2 pieces and subjected to multiple enzymatic digestions until suspensions of contacting ventricle heart cells were isolated and seeded into multipurpose Rose culture chambers. Neonatal rat (1-2 day old) ventricular cells were cultured in Rose chambers using slight modifications of the basic methods described by Mark and Strasser, (1966). The hearts were removed and the atria discarded. The ventricles were cut into small pieces (approximately 1 mm^3) and subjected to stepwise enzymatic digestion in 0.25 Viokase. The cells obtained in each fraction were kept separate rather than being pooled. The supernatants from each fraction were immediately centrifuged at low speed for 5 min. The cell pellet from each fraction was resuspended in a small volume of

minimal essential standard medium and immediately seeded into Rose chambers. The original inoculum varied between 5×10^5 and 1×10^6 cells per chamber. The $CaCl_2$ concentration in this medium was 2.1 mM. Yield and viability of ventricular cells in culture were much improved when cells obtained from each fraction were kept separate.

The Rose chambers were incubated at 37°C and left undisturbed for 2 days. Thereafter, medium was replaced daily with fresh prewarmed medium, and the chambers were returned to the incubator. Medium was exchanged 2 hours before the experimental sessions.

Hydrazine Stock Solutions

Hydrazine (NH_2NH_2, MW 32.05) and monomethyl hydrazine (NH_2NHCH_3, MW 46.09) were supplied through the courtesty of Dr. Ronald Shank (University of California, Irvine). Stock solutions of 100 and 10 mM hydrazine were prepared by dilution into 0.01 N HCI. The stock solutions were diluted from 50- to 1000-fold in tissue culture medium just prior to application on the cultures. Control cultures received a corresponding amount of 0.01 N HCI without hydrazine in the medium. In the quantities used, the addition of 0.01 N HCI with or without hydrazine did not significantly alter the pH of the medium.

Growth Studies

To study the growth effects of hydrazines the specified quantity of cells were plated out in Falcon T-75 flasks in tissue culture medium. Sufficient flasks were prepared such that two flasks were counted to determine each data point for a given hydrazine concentration. At the specificied times, two flasks from the entire population were harvested as follows: the cells in a flask were examined by inverted phase microscopy, washed with fresh medium to remove debris, enzymatically removed from

the flask as previously described (Siemens, et al., 1980), and counted and sized by a Coulter counter-channelyzer (Model ZBi, Coulter Electronics). At this time, the medium in all the remaining flasks was replaced with fresh medium and supplemented with the appropriate concentration of fresh hydrazine stock solution. Control cultures received fresh medium and a corresponding aliquot of 0.01 N HCl without hydrazine. At each subsequent point of harvest, two flasks of cells representing each hydrazine concentration were similarly harvested, and the medium in the remaining flasks was replenished again with fresh medium containing the appropriate concentration of fresh hmdrazine. The medium was changed at different intervals in the various experimental schemes.

Scanning Electron Microscopy

Xenopus cells (75 x 10^3 cells/ml) were plated into Rose chambers in medium containing either 0.01 N HCl with hydrazine (final experimental culture hydrazine concentrations from 0.01 to 1.0 mM) or corresponding amounts of 0.01 N HCl alone (control cultures). Fresh medium containing Hz and MMH was added at 48 hr. after 3 days, the cells were fixed in medium with 2% glutaraldehyde (30 min. at room temperature, and overnight at 4°C), and the adherent cells on the glass coverslips were processed for SEM according to the procedures described by Cohen et al. (1968). The coverslips were rinsed 5 min. in phosphate-buffered saline and run through a series of 5 min. dehydrations (30-100%, Freon 113), critical point drying (Omar SPE-900EX) utilizing Freon 113 and gold evaporation (Technis Hummer II, 3 min. at 10 V). Cover slip specimens were mounted with silver paint on aluminum studs and analyzed on a Hitachi HS500 scanning electron microscope (15-20 kV, tilt angle of 30-55°). Random samples of cells on the cover-slips were examined with regard to the quantity of cell surface projections

(light, moderate, or heavy surface detail) that each cell displayed. Tabulations of the number of cells displaying each type of surface detail were prepared from each specimen by an investigator who was unaware of which specimen corresponded to each hydrazine concentration. Evaluation of the observed data was verified with a Student's t test (confidence level at least 95%).

Analysis of Intracellular Mitochondrial Fluorescence

A new method (Siemens et al., 1982) was employed to study alterations in intra-mitochondrial biochemistry in a living cell. This method involves (1) selective binding of a fluorescent probe for respiratory activity to individual mitochondria in living cells, (2) monitoring the fluorescence emission of this probe while challenging the cells with suspected metabolic inhibitors (in the case of the present study, hydrazines).

Aqueous stock solutions (1 mg/ ml) of Rhodamine 6G were diluted into heart medium to yield a final dye concentration of 1.8 ug/ml. This solution was then injected into Rose chambers containing the heart cell cultures. The cells were exposed to the Rhodamine solutions for 30 min. and rinsed two times for 10 min. with fresh culture medium. One hour after Rhodamine exposure the cells were examined under a standard Zeiss RA epi-fluorescence microscope using a 50 W mercury lamp as a light source. Epi-fluorescent filter sets (Zeiss 437715 or #487709 were used). These filter sets permitted observation of the fluorescent patterns of the entire cell. Upon determination that the mitochondria were strongly fluorescent, the cells were moved to a laser driven fluorescent microscope.

The laser system is similar to that used in membrane photobleaching studies (Kopel et al., 1976). A continuous wave (CW) Helium Cadmium (HeCd)

laser at wavelength 442 nm (output 6-8 mW) was directed into the normal mercury lamp attachment port of a standard epifluorescent Zeiss RA microscope. Prior to entry into the microscope, the laser beam passed through a spatial filter, a recollimating lens, and a set of neutral density filters. The neutral density filters were used to attenuate the laser beam to levels that were high enough to stimulate fluorescence but not so high as to structurally damage the target organelle. Power in the 0.5 μm focused spot varied between 9.2×10^{-8} W - 12.3×10^{-7} W.

The laser was focused into a single mitochondrion that had been preselected by phase contrast observation prior to allowing the laser to enter the microscope. A calibrated reticle in the ocular of the microscope was used to identify the precise location of the focused laser beam. Once the calibration point where the laser was focused was identified, an appropriate target mitochondrion was moved under the point, and the laser was permitted entry into the microscope. An epifluorescent beam splitter that reflects below 510 nm, and transmits above 520 nm, was used to deflect the laser down to a 100X phase Neofluar objective where the beam was focused to a 0.5 μm spot diameter. Wavelengths between 520-560 nm emitted from the target were passed up to a detection system which was a sensitive EMI 9862B/100 photomultiplier tube mounted above the microscope and connected to a Tracor Northern model TN 1710 multichannel analyzer. Using this device it was possible to record precisely the fluorescent intensity by counting photons emitted from the target specimen over selected time periods. No attempt was made to measure different spectral components. The observations were strictly for total fluorescence changes over time between 520-560 nm. After each fluorescence measurement was made, the laser focal point was visually checked to determine if it was still

focused on the exact same point in the mitochondrion. If the cell had moved, or the microscope had gone out of focus, the data were discarded. Above measurements were made for cells treated with Hz, MMH, and untreated controls.

RESULTS

Growth Studies

Monomethyl hydrazine (MMH) has an inhibitory effect on A6 cell growth in a dose-dependent fashion similar to that observed for the parental hydrazine compound (Hz) (Fig. 1). However, MMH appears to be more toxic to cells (1.0 mM of MMH is lethal and 1.0 mM of Hz only retards cell growth). Both compounds are generally cytotoxic above 2 mM.

Cell Surface Studies

As reported earlier (Siemens et al., 1980), scanning EM studies of hydrazine treated cells reveal a direct effect on the amount of cell surface microvilli projections in A6 cells (Figure 2). A comparison of hydrazine and monomethyl hydrazine treated cells (Table 1) demonstrates that both compounds cause a major reduction of microvilli (78% and 79% "light") at 1.0 mM. In addition 0.1 mM treatments with both hydrazines cause a reduction in microvilli; however, the reduction is much greater for Hz (60% light) than MMH (35% light). Notwithstanding this difference, both compounds cause a reduction of microvilli when compared to the control levels.

Mitochondrial Effects

The normal fluorescence patterns of rhodamine-treated heart cell mitochondria can be categorized into three types, A,B,C (Fig. 3). Type A is an irregular oscillating pattern with peaks and valleys of fluorescence

of varying heights and frequencies. Type B is a pattern of a more regular oscillation in which there is frequently a rapid, intense "burst" of fluorescence just prior to each major decrease in fluorescence. Type C is characterized by a lack of oscillation, but contains one major fluorescence peak and a "burst" of fluorescence prior to a single large decrease in fluorescence. All three of these fluorescent patterns can be detected in normal, untreated populations of cells with a frequency of 70% Type A, 20% Type B, and 10% Type C. Treatment with Hz appears to eliminate Type B with Type A occurring in 66% of the cases and Type C, 33%. MMH causes a reduction in Type A to 12% and an increase in Type C to 63%.

DISCUSSION

The results of the growth studies indicate that MMH has an effect similar to that reported for the parental hydrazine compound: a temporary inhibition followed by a return to log phase growth at low concentrations and general cytotoxicity at higher concentrations (above 2 mM).

As indicated by the scanning EM studies, one of the major target sites of both compounds is the cell surface. However, it is interesting to note that the reduction in cell surface microvilli is much more pronounced with Hz than with MMH. Both 0.1 mM and 1.0 mM Hz cause a major reduction of microvilli, whereas 0.1 mM of MMH only reduced microvilli to the "light" condition in 35% of the cases (compared to 17% in the control).

The studies on mitochondrial fluorescence clearly demonstrate that the hydrazines penetrate the cell surface and have either an indirect (through some breakdown product) or direct effect on other membrane organelles. Furthermore, these results support other studies suggesting metabolic

inhibition as a mechanism of hydrazine action (Dost et al., 1979). Of particular interest is the finding that MMH causes a major reduction in the Type A oscillating fluroescence response, and an increase from 10% to 63% in the non-oscillating Type C response. A significant reduction in the Type A oscillating fluorescence pattern was not seen for parental hydrazine. In other studies on the oscillating fluorescence patterns (Siemens and Berns, 1981) a reduction in mitochondrial oscillations was caused by such respiratory inhibitors as oligomycin and valinomycin. These compounds specifically inhibit mitochondrial respiration at different points in the electron transport chain. The results of the present study suggest that MMH has a major effect on mitochondrial respiration. The metabolic results with parental hydrazine are less pronounced. These results are consistent with other studies that demonstrate differences between the effects of methylated hydrazines and parental hydrazine. Rogers and Back (1981) demonstrated thymidine mutagenesis for Hz and several of its derivatives (UDMH, SDMH), but not MMH. Similarly, Schiller et al. (1979) demonstrated differences in the effects of hydrazine and 1, 2 dimethyl hydrazine on the brush border enzymes of developing hamster intestine.

The nature of the differences between the effects of parental hydrazine and monomethyl hydrazine is basic to an adquate understanding of the toxicology of this class of compounds. Combining the methods of biochemistry, structural biology, and biophysics as described here provides a multidisciplinary approach to the pharmacology and toxicology of a class of compounds that is rapidly becoming a major constituent in the environment.

ACKNOWLEDGEMENTS

Supported by a grant from the U.S. Air Force Office of Scientific Research #AFOSR 80-00062A, and NIH Grant HL 15740. Part of this work was conducted in an NIH Biotechnology Resource (LAMP) under grant RRO 1192.

FIGURE LEGENDS

Fig. 1. Growth curves of A6 kidney cells in solution containing hydrazine (Hz) and monomethyl hydrazine (MMH) in the in the indicated concentrations. Cells were exposed to fresh hydrazines at every point along the graph throughout the duration of the experiment.

Fig. 2. Scanning electron micrographs of A6 cells which typify (A) heavy, (B) moderate, and (C) light cell surface projections. Bar, 5 μm.

Fig. 3. Types of fluorescent intensity responses of single mitochondria to hydrazine and monomethyl hydrazine. Small dash at the front of each sequence represents background photon counts prior to exposing the mitochondrion to the laser.

TABLE 1

SEM Analysis of MMH and Hz-treated A-6 cells.

Chambers of A-6 cells were set up and treated for 48 hours with varying concentrations of MMH, Hz. After fixing fixing, the cells were analyzed via SEM for dose-related differences in the cell surface detail. The values in parenthesis are Hz, and not in parenthesis, MMH.

Concentration	Light Detail (%)	Moderate Detail (%)	Heavy Detail (%)	Total# Cells
Control	17 (24)	43 (37)	40 (39)	124 (196)
0.10 mM	35 (60)	40 (31.5)	25 (8.5)	116 (188)
0.5 mM	93	7	0	108
1.0 mM	78 (79)	18 (17)	4 (4)	50 (203)

REFERENCES

1. Kimball, R. F. (1977). The mutagenicity of hydrazine and some of its derivatives. Mutation Res. 39:111-126.

2. Rogers, A. M. and Back, K. C. (1981). Comparative mutagenicity of hydrazine and 3 methylated derivatives in L5178Y mouse lymphoma cells. Mutation Res. 89:321-328.

3. Barkla, D. H. and Tutton, P. J. M. (1978). Ultrastructure of 1, 2-dimethylhydrazine-induced adenocarcinomas in rat colon. Natl. Cancer Inst. 61:1291-1294.

4. Dost, F. N., Johnson, D. E. and Wang, C.-H. (1979). Metabolic toxicity of simple hydrazines: Monomethyl hydrazine (MMH). Toxic. and Appl. Pharm. 49:225-236.

5. Schiller, Carol M., Walden, R. and Kee, T. E., Jr. (1979). Effects of hydrazine and its derivatives on the development of intestinal brush border enzymes. Toxic. and Appl. Pharm. 49:305-311.

6. Greenhouse, G. (1976). Evaluation of the teratogenic effects of hydrazine, methylhydrazine and dimethylhydrazine on embryos of Xenopus laevis, the South African clased toad. Teratology 13:167-178.

7. Siemens, A. E., Kitzes, M. C. and Berns, M. W. (1980). Hydrazine effects on vertebrate cells in vitro. Toxicol. and Appl. Pharm. 55:378-392.

8. Cohen, A. L., Marlow, D. P., and Garner, G. E. (1968). A rapid critical point method using fluorocarbons (freons) as intermediate and transitional fluids. J. Microsc. 7:331-342.

9. Siemens, A. E., Walter, R. J., Liaw, L.-H. and Berns, M.W. (In press). Laser stimulated fluorescence of submicron regions within single mitochondria of Rhodamine treated myocardial cells in culture. Proc. Natl. Acad. Sci., USA.

10. Kopel, D. E., Axelrod, D, Schlessinger, J., Elson, E. L. and Webb, W. W. (1976). Dynamics of fluorescence marker concentration as a probe of mobility. Biophys. J., 16:1315-1329.

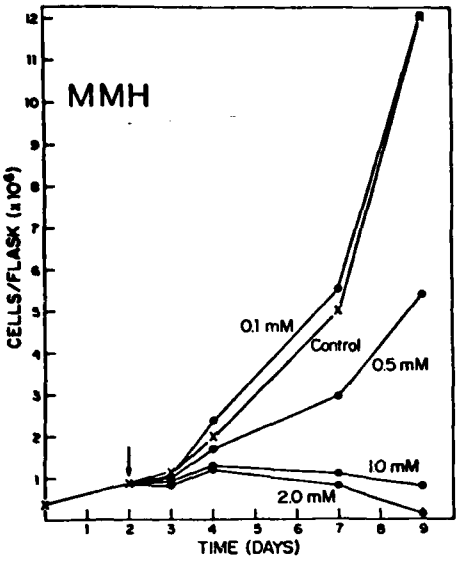


Fig. 1 (right)

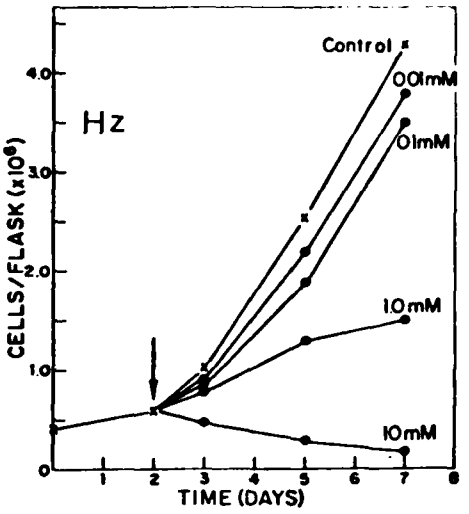
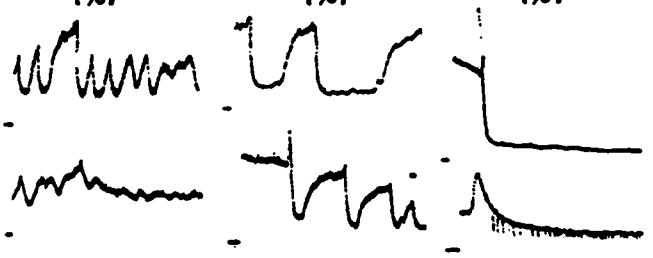


Fig. 1 (left)

Hz & MMH Effects on Fluorescent Patterns Emitted from Rh 6G Treated Heart Cell Mitochondria



Control	**70**	**20**	**10**	**10**
Hz	**66**	**-**	**33**	**9**
MMH	**12**	**25**	**63**	**8**

Fig. 3

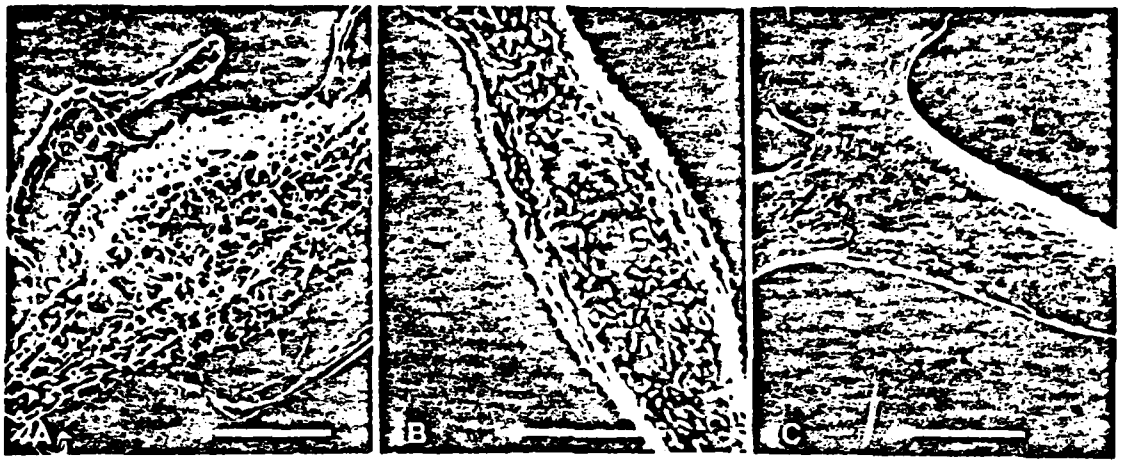


Fig. 2

"D"

Ultrastructural and Electrophysiological Effects of Hydrazines on Cells in Vitro

Michael W. Berns, Ann Elizabeth Siemens, Margarita Kitzes, L.-H. Liaw
University of California, Irvine, CA 92717

In our earlier studies (Siemens et al., J. Tox. and Appl. Pharm. 35:378, 1980) it was demonstrated that the parent hydrazine compound affected the growth rates in vitro of several vertebrate cell types in a dose dependent way. In addition, one of the major target sites of hydrazine was shown to be the cell membrane. The effects on the cell membrane were demonstrated by (1) an increased rate of cell fusion, (2) a dose dependent reduction in cell surface microvilli projections, and (3) electrophysiological effects on cardiac cell membranes.

In an effort to further elucidate the above observations additional studies were undertaken with the parent hydrazine compound (Hz) and the hydrazine derivative, monomethyl hydrazine (MMH). Cell surface microvilli were examined with the scanning electron microscope (SEM) after various exposure times to hydrazine and following a 3 day recovery in control medium. The results demonstrated that at all doses studied (0.01mM to 1.0mM) the effect of the hydrazine on the cell surface appeared to be irreversible even when the cells were in recovery medium for three days. Studies on MMH demonstrated dose dependent growth suppression curves similar to those obtained with Hz. In addition SEM analysis of the cell surface detail also showed a dose related decrease in microvilli. The optimum non-lethal dose for the effects in MMH was 0.5 mM as compared to 1.0 mM for Hz.

The transmission electron microscope (TEM) was employed in order to determine if the hydrazines were having effects on any specific internal organelles. The only organelles that were obviously affected were the mitochondria. The parental hydrazine compound had a subtle effect on the degree of cristae packing. There appeared to be an unusually high number of mitochondria with large spaces between the cristae. However, there appeared to be normal deposits of calcium in the mitochondria. MMH had a much more severe effect on the mitochondria. The mitochondria appeared to be large, swollen, and devoid of any organized cristae arrays. In addition, there were no calcium deposits, and the outer mitochondrial membranes stained very darkly.

Electrophysiological data also demonstrated a distinct difference between Hz and MMH effects on the cell membrane. MMH (1mM) produces characteristic changes in the normal spontaneous intracellular electrical activity and contractility of cultured myocardial cells. During the first 20 min. to 30 min. of continuous exposure to the drug, the cells maintain their normal resting membrane potential levels but exhibit a marked increment in action potential discharge and contraction frequency. The occurrence of depolarizing after potentials is characteristic during this time period and may lead to irregularities in discharge pattern. A progressive decrement in action potential discharge frequency to rates below control levels (prehydrazine) ensues during the following 30 min. to 60 min. of continuous drug exposure. Within this time period, the cells become progressively hyperpolarized to higher than normal levels of resting membrane potential. Interspike intervals become increasingly prolonged with eventual severe reduction of action potential discharge and stabilization of resting membrane potential at a hyperpolarized level. Electrical and contractile activities return to normal within 15 min. following replacement of experimental with normal medium. These results suggest that MMH may mediate an increase in $[Ca]_i$ which would be responsible for the observed increase in K^+ conductance. The parent hydrazine compound (Hz) produced increased depolarizations at 1mM as compared to hyperpolarization produced by the MMH. These results suggest different ionic effects of the two compounds.


DA
FILM
7